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Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography

(bone matrix/alkaline phosphatase/growth factors/calcium/cartilage)

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ABSTRACT Implantation of demineralized diaphyseal bone matrix in subcutaneous sites induces a sequence of events resulting in the local differentiation of endochondral bone. Demineralized bovine bone matrix was dissociatively extracted in 4.0 M guanidine hydrochloride and the bone-inductive proteins were purified >12,000-fold. The purification steps include affinity chromatography on heparin-Sepharose, hydroxyapatite chromatography, gel filtration, and C_{18} reverse-phase HPLC. Since the purified protein in conjunction with insoluble collagenous bone matrix induced new bone differentiation *in vivo* we have designated this component osteogenin. The osteogenic potential is specific for osteogenin and is not exhibited by previously isolated growth factors.

The remarkable potential for regeneration and repair in bone has been known from the days of Hippocrates in ancient Greece. It has been suspected that in bone there may be a substance, osteogenin, that might initiate bone growth (1). Implantation of demineralized diaphyseal bone matrix in intramuscular and subcutaneous sites results in local bone differentiation (2, 3), and it is possible osteogenins are present in the matrix. The sequential developmental cascade in response to the implantation of matrix consists of the following major phases: chemotaxis and attachment of mesenchymal stem cells to the matrix, proliferation of progenitor cells, and differentiation of cartilage, bone, and hematopoietic marrow (3–5). Progress in the isolation of osteogenins has been slow due to the fact that bone matrix is in the solid state. We have shown that the endochondral bone-differentiation activity of bone matrix could be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone-induction activity (6). This advance provided a method for assaying soluble components for their ability to induce endochondral bone differentiation *in vivo* and permitted their further purification. The putative differentiation factors have a molecular mass of <50 kDa (6), appear to be homologous in several species of mammals (7), stimulate fibroblast proliferation (8), and transform mesenchymal cells derived from muscle into chondrocytes *in vitro* (9). Aggregates of the bone morphogenetic protein and other proteins from bovine demineralized matrix induce formation of new bone (10). The osteoinductive potential of demineralized bone matrix was inhibited by pretreatment with heparinized plasma (11) and heparin (12), possibly by binding to the active sites in the matrix. In view of this we have explored the utility of heparin affinity columns to purify bone-inductive protein. We report here the isolation of bone-inductive protein, osteogenin from bovine demineralized bone matrix, using heparin affinity chromatography and reverse-phase HPLC.

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MATERIALS AND METHODS

Dissociative Extraction and Ethanol Precipitation. Dehydrated diaphyseal bovine bone matrix powder (particle size, 74–420 μm) was demineralized. Two hundred grams of acid-demineralized bovine bone matrix (obtained from 1 kg of mineralized matrix) was extracted dissociatively in 4 M guanidine hydrochloride/50 mM Tris·HCl, pH 7.0, containing protease inhibitors at 4°C for 16 hr (6). The extract was centrifuged at 20,000 × *g* for 30 min at 4°C, and the supernatant was collected and concentrated using a YM-10 membrane. The proteins in the concentrated extract were ethanol precipitated by adding 170 ml of cold (−20°C) absolute ethanol to 20 ml of concentrate and kept cold at −20°C for 30 min. The precipitate was obtained upon centrifugation at 4°C, 10,000 × *g* for 30 min, and the pellet was washed three times in 85% ethanol and lyophilized.

Heparin-Sepharose Chromatography. Two and one-half grams of lyophilized protein obtained by ethanol precipitation was dissolved in 400 ml of 6 M urea in 50 mM Tris·HCl (pH 7.0) (buffer A) containing 0.1 M NaCl and clarified by centrifugation at 20,000 × *g* for 30 min. The supernatant was stirred for 15 min with 200 ml of hydrated heparin-Sepharose (Pharmacia) equilibrated with buffer A containing 0.1 M NaCl and then packed in a column (2.6 × 40 cm). The column was washed with 3 vol of initial buffer and was eluted sequentially with buffer A containing 0.15 M NaCl and 0.5 M NaCl. The activity was confined to 0.5 M NaCl fraction and was used for further purification.

Hydroxyapatite-Ultrogel Chromatography. A 150-ml pool of proteins eluted by buffer A containing 0.5 M NaCl from heparin-Sepharose was applied directly to a column (2.6 × 20 cm) of hydroxyapatite-Ultrogel (LKB, Instruments) equilibrated with buffer A containing 0.5 M NaCl and 10 mM Na_2HPO_4. The unadsorbed protein was collected and the column was washed with 3 column vol of buffer A containing 0.5 M NaCl and 10 mM Na_2HPO_4. The column was subsequently eluted stepwise with buffer A containing 100 mM, 200 mM, and 500 mM Na_2HPO_4. The protein peak eluted by 100 mM Na_2HPO_4 was dialyzed in a 3500 M_r cutoff bag (Spectropor) against distilled water and lyophilized. In some experiments the 100 mM phosphate eluate was run a second time on a heparin-Sepharose column as described above.

TSK SW3000/SW2000 Gel-Exclusion Chromatography. TSK SW3000 (8 mm × 300 mm) and TSK SW2000 (8 mm × 300 mm) columns obtained from Beckman were connected in tandem, attached to a precolumn (8 mm × 100 mm), and equilibrated with 4 M guanidine hydrochloride/50 mM Tris·HCl, pH 7.0. Thirty milligrams of the lyophilized protein

Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TGF-β, transforming growth factor type β; FGF, fibroblast growth factor; CDGF, cartilage-derived growth factor.

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from hydroxyapatite-Ultrogel was dissolved in 600 μl of 4 M guanidine hydrochloride/0.05 M Tris·HCl, pH 7.0, and the solution was clarified by centrifugation. Two hundred microliters containing ≈10 mg of protein was loaded and eluted with 4.0 M guanidine hydrochloride/50 mM Tris·HCl, pH 7.0, of a flow rate of 0.25 ml/min. Fractions were collected and bioassayed individually; fractions 19–21 were osteoinductive and were ethanol precipitated and rechromatographed on the same column and obtained as a single peak. The sample was dialyzed against distilled water and lyophilized.

Reverse-Phase HPLC. Final purification was achieved on a μBondapak C_{18} silica-based HPLC column (particle size, 10 μm; pore size, 300 Å; Waters Associates, Milford, MA) equilibrated with 0.1% trifluoroacetic acid in 10% acetonitrile. The lyophilized biologically active preparation (2 mg) obtained from TSK SW3000/SW2000 gel-exclusion chromatography was dissolved in 1 ml of 0.1% trifluoroacetic acid/10% acetonitrile and centrifuged in a Microfuge. The sample was loaded and washed in 0.1% trifluoroacetic acid/10% acetonitrile over 20 min, and the bound proteins were eluted with a linear gradient of 10–30% acetonitrile in 0.1% trifluoroacetic acid over 30 min, 30–50% acetonitrile in 0.1% trifluoroacetic acid over 60 min, and 50–70% acetonitrile in 0.1% trifluoroacetic acid over 20 min at 22°C. The flow rate was 0.25 ml/min, and 0.5-ml samples were collected, lyophilized, bioassayed for bone-differentiation activity, and characterized by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Bioassay. Fractions obtained from various steps of purification were bioassayed for bone-differentiation activity by reconstituting a portion of the fraction with samples of 4 M guanidine hydrochloride-extracted demineralized rat bone matrix residue (6, 7). Demineralized bone matrix of rat (control) and the various reconstituted bone matrix preparations were bioassayed by subcutaneous implantation into male Long–Evans rats at bilateral sites located over the ventral thorax (3). Alkaline phosphatase activity and calcium content of the mineralized implant were determined 12 days after implantation as indices of bone formation as described (3, 13). The histological appearance of the implants was examined by fixing the implants in Bouin's fixative and embedding in JB4 plastic medium. One-micrometer sections were cut and stained with toluidine blue.

Polyacrylamide Gel Electrophoresis. Protein fractions obtained during the purification steps were characterized by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (6). Gradient gels (5–20%) and 15% gels were used as the separating gel with a spacer gel of 3%. Samples were heated to 90°C for 3 min with or without dithiothreitol. The gels were glutaraldehyde fixed and silver stained (Bio-Rad kit).

Growth Factors. Well-characterized growth factors and hormones were used in an experiment to assess the specificity of the osteogenin action. These factors were gifts and included platelet-derived growth factor (PDGF), G. R. Grotendorst (14); epidermal growth factor (EGF) (15), Collaborative Research, Waltham, MA; transforming growth factor type β (TGF-β) (16), Anita Roberts and M. Sporn; fibroblast growth factor (FGF) (17), D. Gospodarowicz; cartilage-derived growth factor (CDGF) (18), M. Klagsbrun; insulin, Sigma. Bovine growth hormone was a gift of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases Hormone Distribution Program of the National Institutes of Health. The various growth factors were examined in the reconstitution bioassay for bone formation in doses ranging from 0.5 μg to 5.0 μg depending on the availability of the factor.

RESULTS

Purification. One kilogram of mineralized bovine diaphyseal bone powder on acid demineralization yielded ≈200 g of demineralized bone matrix. Dissociative extraction of demineralized matrix by 4 M guanidine hydrochloride and subsequent ethanol precipitation yielded 7.5 g of protein.

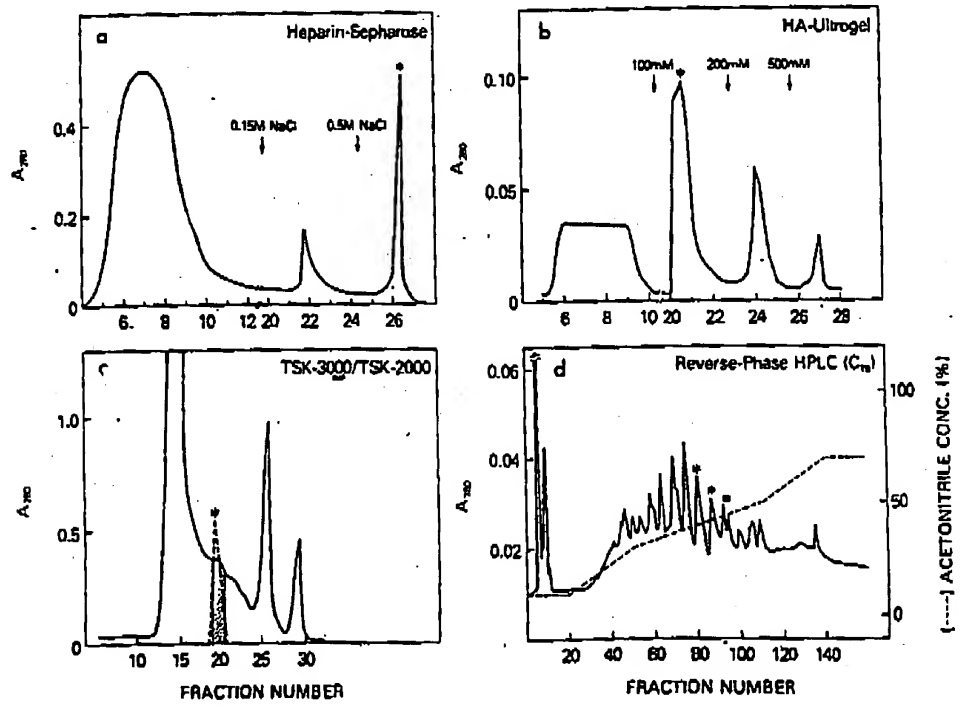

FIG. 1. Purification of osteogenin. Sequential chromatography of dissociative extract of demineralized bone matrix on a heparin-Sepharose column (*a*), a hydroxyapatite-Ultrogel column (*b*), a HPLC TSK SW3000/SW2000 molecular sieve column (*c*), and a reverse-phase HPLC C_{18} column (*d*). The asterisks indicate the peaks with osteogenic activity.

Table 1. Alkaline phosphatase activity and calcium content of day-12 implants of various protein fractions reconstituted with inactive collagenous residue

Group	Reconstitution, μg of protein per 25 mg of rat residue	Alkaline phosphatase, units/mg of protein	Calcium content, μg/mg of tissue
Rat demineralized bone matrix (control)	—	1.48 ± 0.30	4.47 ± 1.46
Inactive collagenous residue of rat	—	0.06 ± 0.01	0.25 ± 0.03
Heparin-bound fraction (0.5 M NaCl eluate)	250	2.40 ± 0.52	7.41 ± 1.86
Hydroxyapatite-bound fraction (100 mM Na_2HPO_4 eluate)	125	2.68 ± 0.12	13.45 ± 2.73
TSK SW3000/SW2000 fraction (fractions 19–21)	25	2.35 ± 0.06	6.26 ± 1.32
Reverse-phase C_{18}			
Peak 9	5	4.11 ± 0.76	22.95 ± 8.99
Peak 10	4	1.84 ± 0.31	4.46 ± 2.24
Peak 11	4	1.63 ± 0.31	2.86 ± 0.24

Values are mean ± SEM of four observations. Similar results were obtained in three other experiments.

Ethanol-precipitated, lyophilized protein was subjected to affinity chromatography on heparin-Sepharose as shown in Fig. 1*a*. Most of the protein (about 95%) was unbound; ≈3% was eluted as a biologically active fraction by 0.5 M NaCl. The unbound fraction and the fraction eluted by 0.15 M NaCl had no bone-induction activity when they were bioassayed as such or after further purification through Sepharose CL-6B. The 0.5 M NaCl eluate from the heparin-Sepharose column was pumped directly to the hydroxyapatite-Ultrogel column. The bound bone-inductive proteins were eluted by 100 mM Na_2HPO_4 (Fig. 1*b*). The components eluted by 100 mM Na_2HPO_4 were shown to induce endochondral bone, as measured by the alkaline phosphatase activity, calcium content, and histology (Table 1). Gel-exclusion chromatography of the 100 mM phosphate eluate from hydroxyapatite-Ultrogel fractionated on a TSK SW3000/SW2000 column is shown in Fig. 1*c*. Of the fractions bioassayed, only 19–21 resulted in bone induction; fraction 20 had highest activity, as measured by alkaline phosphatase activity, calcium content, and histology (data not shown). When all three fractions were pooled and rechromatographed, a single symmetric protein peak that induced new bone on bioassay (Fig. 1*c* and Table 1) was obtained.

The endochondral bone-differentiation activity obtained from the TSK column was chromatographed on a C_{18} silica-based reverse-phase HPLC μBondapak column (Fig. 1*d*). Among the various peaks, only peaks 9–11 that eluted between 35% and 45% acetonitrile concentration induced endochondral bone *in vivo*. Of the three peaks, peak 9 had the highest specific activity (Table 2). Further purification of this peak was achieved by reverse-phase C_{18} column chromatography. Electrophoresis of the active fraction 9 revealed a diffuse band with an apparent molecular mass of about 22 kDa (Fig. 2). The histological appearance of the implant in response to peak 9 as shown in Fig. 3 reveals new bone.

Specificity. The osteogenic potential of the newly isolated osteogenin raised the question about the specificity of the response. To address this question, several purified growth factors such as PDGF, EGF, TGF-β, FGF, and CDGF and hormones such as insulin and pituitary growth hormone were tested in the *in vivo* reconstitution assay for osteogenesis. As summarized in Table 3, among the factors tested to date only osteogenin initiated new bone differentiation.

DISCUSSION

The present data represent a rapid and reproducible method for purification of osteogenin from demineralized extracellular matrix of bone. The method is based on affinity for heparin and hydroxyapatite and reverse-phase HPLC. One to 5 μg of osteogenin evoked bone formation in the present work as compared to 1–5 mg of bone morphogenetic protein (10). The occurrence of osteogenic activity in more than one peak indicates microheterogeneity or possible degradation during

Table 2. Purification of osteogenin

Purification step	Protein, mg	Biological activity,* units	Specific activity, units/mg of protein	Purification, fold
Ethanol precipitation	7500†	1237‡	0.165	1
Heparin-Sepharose	225†	2983	13.26	80
Hydroxyapatite	60†	2888	48.14	292
TSK SW3000/SW2000	6†	672	112.0	679
Reverse-phase C_{18}				
Peak 9	0.03§	61.6	2052.0	12,436
Peak 10	0.02§	10.0	500.0	3,030
Peak 11	0.02§	6.3	317.5	1,924

Values are based on 1 kg of bovine bone matrix (200 g of demineralized matrix).

*One unit of osteogenic activity is defined as the amount that exhibits half-maximal bone-differentiation activity compared to rat demineralized bone matrix, as determined by calcium content of the implant on day 12 in rats.

†Proteins were measured by the Lowry (19) method.

‡Ethanol-precipitated guanidine extract of bovine bone is a weak inducer of bone in rats on reconstitution, possibly due to endogenous inhibitors (7). This precipitate was subjected to molecular sieve chromatography on Sepharose CL-6B and proteins <50 kDa were separated and used for bioassay (6–7).

§Protein was estimated by a dye-binding method (Bio-Rad) and using absorbance values at 280/220 nm.

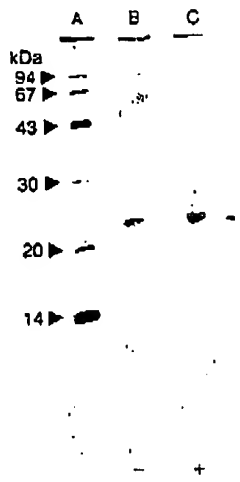

FIG. 2. Silver-stained NaDodSO$_4$/polyacrylamide gel of the purified osteogenin. Aliquots of reverse-phase HPLC C_{18}-purified osteogenin, peak 9 (~1 μg), were analyzed in 15% acrylamide gels with a stacking gel of 3% in nonreducing conditions (−, lane B) and after reduction with dithiothreitol (+, lane C). Molecular mass markers are given in kDa in lane A.

purification. TGF-β was recently isolated from bovine bone matrix (20, 21) and shown to increase the synthesis of cartilage proteoglycans and type II collagen by muscle-derived mesenchymal cells *in vitro*. However, its role *in vivo* is not clear. In the present investigation TGF-β was tested *in vivo* and found not to induce cartilage or bone. Also it is noteworthy that cartilage (18) and bone matrix (22) have several heparin-binding growth factors, as assayed *in vitro* on mouse 3T3 fibroblasts. For example, bovine bone matrix has factors related to PDGF and anionic and cationic FGF (22), and the CDGF is similar to cationic FGF (23). Pituitary growth hormone has profound effects on bone formation (13). None of the highly purified proteins such as EGF, FGF, CDGF, insulin, and growth hormone demonstrated osteoinductive potential. In recent experiments recombinant bovine growth hormone and recombinant human insulin-like growth factor I were tested singly and in combination at doses ranging from 1 to 5 μg and found to be inactive in the *in vivo* osteogenic assay (A.H.R., unpublished observations). To date, only osteogenin has demonstrated osteogenic potential. The *in vivo* bioassay may prove to be useful in the quest for other osteotropic factors and perhaps chondrogenic substances.

Table 3. Specificity of the osteogenin response

Factor	Alkaline phosphatase,* units/mg of protein	Calcium, μg/mg
Osteogenin	2.22 ± 0.3	13.4 ± 1.8
PDGF	0.11 ± 0.03	ND
EGF	0.14 ± 0.05	ND
TGF-β	0.22 ± 0.07	ND
FGF	0.14 ± 0.06	ND
CDGF	0.16 ± 0.04	ND
Insulin, porcine	0.11 ± 0.04	ND
Growth hormone, bovine	0.12 ± 0.02	ND
None (control)	0.07 ± 0.02	ND

ND, not detectable.
*Values are mean ± SEM of four observations.

The heparin-binding property of osteogenin may explain the earlier observation that pretreatment of demineralized bone matrix by soluble heparin inhibits bone formation (12). It is conceivable that extracellular collagenous matrix may serve as a repository of osteogenin that initiates bone repair locally at the site of bone fracture by way of a controlled release by limited proteolysis by granulocyte proteases (5). This prototypic model demonstrates that extracellular bone matrix in addition to being a passive skeletal framework is a dynamic structure with a bank of latent osteogenin that can be called upon by physiological signals to initiate repair and remodeling. The action of osteogenin may be modulated by other local growth factors and systemic regulators such as hormones and nutrition (24). The availability of purified osteogenin will greatly facilitate the elucidation of the structure and mechanism of action of this differentiation factor as well as molecular cloning and expression of osteogenin by recombinant DNA methodology. Finally, these advances may lead to application of osteogenin in a suitable delivery

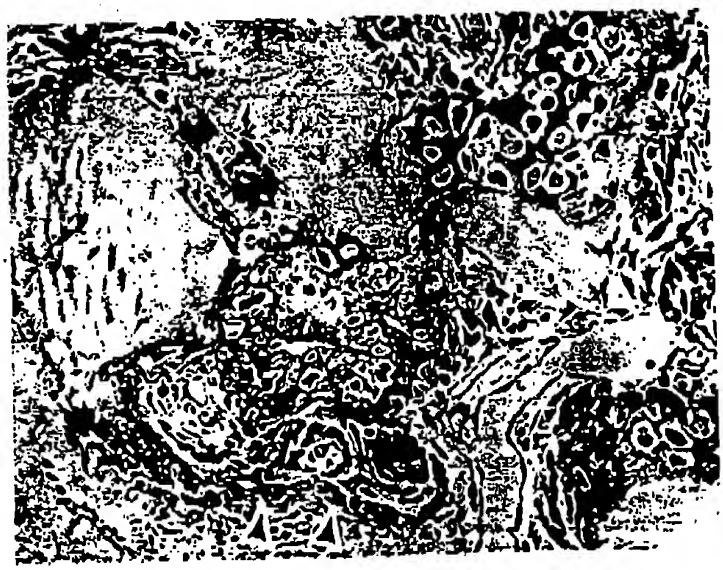

FIG. 3. Photomicrograph of the implant after reconstitution peak 9 of reverse-phase C_{18} HPLC column with inactive collagenous bone matrix on day 12. Bone formation and vascular invasion are evident. The arrowheads indicate osteoblasts. (×220.)

system to correct acquired and congenital craniofacial (25) and skeletal defects and to promote bone healing in non-union of fractures.

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